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β-Lactamases as fully efficient enzymes

Determination of all the rate constants in the acyl-enzyme mechanism

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The rate constants for both acylation and deacylation of β-lactamase PC1 from *Staphylococcus aureus* and the RTEM β-lactamase from *Escherichia coli* were determined by the acid-quench method [Martin & Waley (1988) Biochem. J. **254**, 923–925] with several good substrates, and, for a wider range of substrates, of β-lactamase I from *Bacillus cereus*. The values of the acylation and deacylation rate constants for benzylpenicillin were approximately the same (i.e. differing by no more than 2-fold) for each enzyme. The variation of $k_{cat.}/K_m$ for benzylpenicillin with the viscosity of the medium was used to obtain values for all four rate constants in the acyl-enzyme mechanism for all three enzymes. The reaction is partly diffusion-controlled, and the rate constant for the dissociation of the enzyme–substrate complex has approximately the same value as the rate constants for acylation and deacylation. Thus all three first-order rate constants have comparable values. Here there is no single rate-determining step for β-lactamase action. This is taken to be a sign of a fully efficient enzyme.

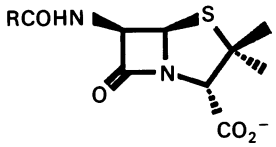
INTRODUCTION

The aim of mechanistic studies on enzymes is to characterize the intermediates on the pathway between substrate and product. The characterization has to be both structural and kinetic. An example of the former is the crystallography and cryoenzymology of ribonuclease (Douzou & Petsko, 1984), and of the latter the work on triose-phosphate isomerase (Albery & Knowles, 1976*a*). β-Lactamases, enzymes that are clinically important for the part that they play in the resistance of pathogens to β-lactam antibiotics, have also been much studied mechanistically (Waley, 1988). The acyl-enzyme mechanism (Scheme 1), involving an active-site serine residue, is now well established for β-lactamases, except those that require metal ions for activity. Full kinetic characterization of the acyl-enzyme mechanism entails measurement of all the four rate constants of Scheme 1, namely k_{+1}, k_{-1}, k_{+2} and k_{+3}. The present paper describes how, building on our earlier work (Martin & Waley, 1988), we have now achieved this for three important class A β-lactamases (Ambler, 1980; Campbell *et al.*, 1989).

The first is β-lactamase I from *Bacillus cereus*, an enzyme whose structure (Samraoui *et al.*, 1986) and site-directed mutagenesis (Madgwick & Waley, 1987) are being studied. The second is the PC1 β-lactamase from *Staphylococcus aureus*, an enzyme whose structure (Herzberg & Moult, 1987) and folding pathway (Mitchinson & Pain, 1985) have been investigated. The third is the RTEM β-lactamase from *Escherichia coli*, which has been much studied mechanistically (Fisher *et al.*, 1980) and by site-directed mutagenesis (Sigal *et al.*, 1984; Schultz & Richards, 1986). The amino acid sequences (Ambler, 1980) of these enzymes are similar,

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} E\text{-acyl} \xrightarrow{k_{+3}} E + P$$

Scheme 1.



R–	Name
$C_6H_5CH_2$–	Benzylpenicillin
$C_6H_5OCH_2$–	Phenoxymethylpenicillin
$C_6H_5OCH(Me)$–	Phenethicillin
$C_6H_5OCH(Et)$–	Propicillin
$C_6H_5CH(CO_2^-)$–	Carbenicillin

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as are the three-dimensional structures of the first two, and the key active-site groups may be presumed to have the same roles. Comparison of all the rate constants for all these enzymes has been particularly illuminating: the enzymes have apparently evolved to nearly optimum efficiency. Finally, work on β-lactamase I has been extended to a range of substrates, whose structures are shown.

MATERIALS AND METHODS

Enzymes

β-Lactamase I from *B. cereus* 569H/9 was purified as described previously (Davies *et al.*, 1974; Baldwin *et al.*, 1980). Its concentration was determined either by activity towards the substrate nitrocefin (Martin & Waley, 1988), or from ϵ_{280} 28000 $M^{-1} \cdot cm^{-1}$. β-Lactamase PC1 from *S. aureus* was from Division of Biotechnology, P.H.L.S. Centre for Applied Microbiology and Research, Porton Down, Wilts., U.K.; concentrations of enzyme solutions were determined either from ϵ_{277} 19500 $M^{-1} \cdot cm^{-1}$ (Carrey & Pain, 1978) or from the initial velocity of nitrocefin hydrolysis at relatively high substrate concentrations (e.g. 25 μM; K_m = 0.26 μM); the $k_{cat.}$ was 5.04 s^{-1} at 30 °C in 50 mM-sodium phosphate buffer, pH 7, containing 0.5 M-NaCl and 0.1 mM-EDTA. The RTEM β-lactamase from *E. coli* was a gift from Professor J. H. Richards.

Substrates

Substrates were quantified by change in absorbance at 240 nm on enzymic hydrolysis. The corresponding Δε values had been determined by measuring the absorbance changes corresponding to pH-stat measurements of substrate proton liberation upon hydrolysis. Resulting values (means of at least three determinations) at pH 7.0 were: benzylpenicillin, 515 $M^{-1} \cdot cm^{-1}$; phenoxymethylpenicillin, 486 $M^{-1} \cdot cm^{-1}$; phenethicillin, 514 $M^{-1} \cdot cm^{-1}$; D-propicillin, 485 $M^{-1} \cdot cm^{-1}$; L-propicillin, 535 $M^{-1} \cdot cm^{-1}$; carbenicillin, 664 $M^{-1} \cdot cm^{-1}$.

Viscous solutions

Buffers of pH 7.0 containing 10 mM-sodium phosphate, 0.1 M-NaCl, 10 μM-EDTA, and various concentrations of sucrose (analytical reagent; East Anglia Chemicals, Ipswich, U.K.) or glycerol as a viscosogen were prepared to a known final concentration based on weight. The relative viscosities, corresponding to the concentrations of sucrose used, were calculated from published values (Weast, 1978).

Experiments in mixtures of ^2H_2O and ^1H_2O

Benzylpenicillin, at 10 times the desired concentration in the enzymic reaction, was dissolved in 100 mM-sodium phosphate buffer, pH 7, containing 1 M-NaCl and 100 μM-EDTA. Then 0.1 ml of this solution was added to x ml of ^1H_2O (where $0 \leqslant x \leqslant 0.9$) and $(0.9-x)$ ml of ^2H_2O. The rate in this region is relatively pH-(and pD-)independent, and the pK_a values of β-lactamase I were raised by about 0.5, comparable with that of the buffer (Hardy & Kirsch, 1984*b*).

Steady-state experiments

The parameters for the hydrolysis of penicillins were determined from analysis by the half-time method (Wharton & Szawelski, 1982) of progress curves obtained by monitoring absorbance changes at 240 nm or 232 nm in a Cary 219 spectrophotometer. If the bandwidth at 232 nm exceeded 2 nm the Δε decreased from 1125 $M^{-1} \cdot cm^{-1}$. All experiments were carried out at 20 °C in 10 mM-sodium phosphate buffer, pH 7, containing 0.1 M-NaCl and 10 μM-EDTA. The presence of lysozyme [0.2 mg/ml (Persaud *et al.*, 1986)] increased the reproducibility of steady-state experiments with PC1 β-lactamase and with β-lactamase I, and did not lead to depletion of substrate.

Stopped-flow experiments

These were carried out at 20 °C in a Hi-Tech SF-42 apparatus (Hi-Tech Scientific, Salisbury, Wilts., U.K.) with automatic data acquisition and signal averaging.

Quenched-flow experiments

These were carried out as described previously (Martin & Waley, 1988), usually with initial concentrations of substrate of 1.5 mM and enzyme of 1 μM. Acyl-enzyme was measured spectrophotometrically at 282 nm by the penamaldate reaction. The time taken for the absorbance to become constant could be reduced by outgassing the samples for about 2 min. The penamaldate assay was repeated at least ten times. In the penamaldate reaction, the Δε values used were: benzylpenicillin, 18800 $M^{-1} \cdot cm^{-1}$; phenoxymethylpenicillin, 18600 $M^{-1} \cdot cm^{-1}$; D-propicillin, 17900 $M^{-1} \cdot cm^{-1}$; L-propicillin, 17900 $M^{-1} \cdot cm^{-1}$; carbenicillin, 17900 $M^{-1} \cdot cm^{-1}$; ampicillin, 22800 $M^{-1} \cdot cm^{-1}$. These values were obtained by titration of $HgCl_2$ from a solution of known concentration to a solution of unknown concentration of acyl-enzyme and noting the corresponding absorbance changes at 282 nm. [^{3}H]Benzylpenicillin {[*phenyl*-4(n)-^{3}H]benzylpenicillin; 951 GBq/nmol; from Amersham International, Amersham, Bucks., U.K.} (sp. radioactivity 867 c.p.m./nmol in the reaction mixture) was used as described above; the protein bound to the Eppendorf tube (Sarstedt, W. Germany) was washed with water four times and then dissolved in 0.2 ml of 1% SDS in 0.1 M-sodium acetate buffer, pH 4.5, and 0.8 ml of scintillation fluid (Unisolve E; Koch–Light, Haverhill, Suffolk, U.K.).

Interpretation of solvent kinetic isotope effects

The solvent isotope effects on a single step in an enzymic reaction may be investigated by proton inventories and analysed by application of the Gross–Butler equation (Schowen & Schowen, 1982). The results are expressed in terms of k_0, the rate constant in ^1H_2O, and k_n, the rate constant in solvent containing atom fraction n of ^2H_2O. In outline, k_n/k_0 is a linear function of n if a single proton is 'in flight' in the reaction being considered, whereas instead $(k_n/k_0)^{0.5}$ is a linear function of n if two protons are 'in flight'. These conclusions apply to a single step of the enzymic reaction; with, for example, serine or cysteine proteinases, synthetic substrates are chosen so that either acylation or deacylation is rate-determining. When neither is rate-determining, two steps have to be taken into account, and, if a single proton is 'in flight' and ground-state fractionation factors are unity, the appropriate equation is:

$$\frac{k_0}{k_n} = \frac{w_2}{1-n+n/k_{+2}} + \frac{w_3}{1-n+n/k_{+3}} \qquad (1)$$

Here w_2 and w_3 are weighting factors for the relative importance of the transition states for acylation and deacylation in ^1H_2O.

RESULTS

Further tests of the method of determining the acyl-enzyme

The use of [^{3}H]benzylpenicillin provided an alternative method of determining the acyl-enzyme; values of F, the fraction of enzyme present as acyl-enzyme in the steady-state, of 0.75 for β-lactamase I and 0.855 for PC1 β-lactamase were obtained. These values were somewhat higher than those obtained by the penamaldate reaction, of 0.51 for β-lactamase I and 0.64 for PC1 β-lactamase. The discrepancy could be due to incomplete removal of low-M_r radioactive material on the one hand, or instability of the penamaldyl derivative on the other. The differences were not large, but the values of k_{+2} and k_{+3} are rather sensitive to such differences. For example, the change noted above for PC1 β-lactamase (0.855 to 0.64) would result in a decrease of about 25% in k_{+3} and an approximate doubling of k_{+2}. That both methods were indeed measuring the fraction of enzyme present as acyl-enzyme is convincingly shown by the variation of F/F_∞ with initial concentration of benzylpenicillin (Fig. 1), where F_∞ is defined as $F(1+K_m/s)$ and s is the substrate concentration at time of quenching (0.12 s). The calculated values of F/F_∞ at different concentrations of benzylpenicillin in Fig. 1 were obtained from a BASIC program for non-linear regression that utilized the version of the simplex algorithm in Press *et al.* (1986); s was obtained by Duggleby's (1986) method. Thus F/F_∞ was the dependent variable, the initial concentration of benzylpenicillin was the independent variable and K_m and $k_{cat.}$ were parameters. The values of K_m and $k_{cat.}$ thus estimated (114 μM and 1930 s^{-1} respectively) did not differ greatly from the values (73 μM and 1920 s^{-1} respectively) obtained from progress curves. Moreover, the variation of the fraction of enzyme present as acyl-enzyme (in the steady state) with a concentration of benzylpenicillin was the same for both methods (Fig. 1). In practice, the penamaldate method was mostly used because it is less expensive and can be applied to different penicillins. Once a value for F_∞ is known, then k_{+2}/k_{+3} can be found directly as $F_\infty/(1-F_\infty)$.

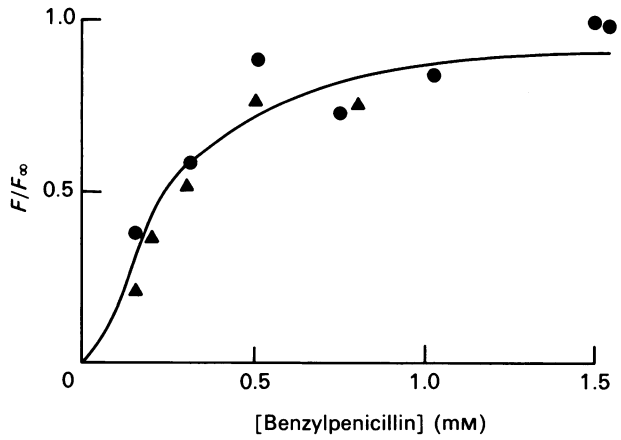

Fig. 1. Dependence of fraction of enzyme present as acyl-enzyme on concentration of substrate

The Figure shows the variation of F/F_∞ with initial concentration of benzylpenicillin; F is the fraction of β-lactamase I present as acyl-enzyme in the steady-state, and F_∞ is the corresponding value at infinite initial concentration of substrate. The extent of labelling was obtained by the use of radioactive benzylpenicillin (●) or of the penamaldate reaction (▲). The curve is theoretical, calculated as described in the text.

PC1 β-lactamase and benzylpenicillin

The penamaldate method gave $F_\infty = 0.64 \pm 0.04$, and hence $k_{+2}/k_{+3} = 1.8 \pm 0.3$; the individual values are given in Table 1.

RTEM β-lactamase and benzylpenicillin

The penamaldate method gave $F_\infty = 0.65 \pm 0.1$, and thus $k_{+2}/k_{+3} = 1.9 \pm 0.7$; the individual values are given in Table 1.

Table 1. Rate constants for the hydrolysis of benzylpenicillin

Progress curves were measured spectrophotometrically at pH 7 at 20 °C for the steady-state parameters $k_{cat.}$ and K_m, whereas k_{+2} and k_{+3} were obtained from acid-quench experiments, and k_{+1} and k_{-1} were obtained from experiments in the presence of sucrose or glycerol. The β-lactamase from *S. aureus* is shown as PC1 β-lactamase and the β-lactamase from *E. coli* as RTEM β-lactamase. The values are given as means ± S.E.M. derived from at least three experiments.

Rate constant (unit)	β-Lactamase I	PC1 β-lactamase	RTEM β-lactamase
k_{+1} ($\mu M^{-1} \cdot s^{-1}$)	41 ± 3	22 ± 2	123 ± 10
k_{-1} (s^{-1})	2320 ± 700	196 ± 30	11800 ± 1500
k_{+2} (s^{-1})	4090 ± 210	173 ± 10	2800 ± 300
k_{+3} (s^{-1})	3610 ± 200	96 ± 10	1500 ± 200
$k_{cat.}$ (s^{-1})	1920 ± 60	62 ± 3	980 ± 100
$k_{cat.}/K_m$ ($\mu M \cdot s^{-1}$)	26.2 ± 4	10.3 ± 1	23.6 ± 5
K_m (μM)	73 ± 5	6.0 ± 1	42 ± 5
K_s (μM)	57 ± 14	8.9 ± 2	96 ± 9

Determination of k_{+1} and k_{-1} for benzylpenicillin

The value of $K_m/k_{cat.}$ for benzylpenicillin with both PC1 β-lactamase and β-lactamase I increased sharply in solutions made more viscous by adding sucrose (Fig. 2). The linear relationship (Nakatani & Dunford, 1979; Cleland, 1986) is:

$$K_m/k_{cat.} = \eta_{rel.}/k_{+1} + (k_{-1}/k_{+2})/k_{+1} \quad (2)$$

and thus k_{+1} may be found from the slope and k_{-1}/k_{+2} from the intercept. The values now found for the rate constants for the first step in the hydrolysis of benzylpenicillin, together with the values of the other rate constants previously found for β-lactamase I (Martin & Waley, 1988), are given in Table 1, as are the rate constants for acylation and deacylation of benzylpenicillin with PC1 β-lactamase. Note that all the first-order rate constants for each enzyme are of the same order of magnitude. However, the absolute values of the first-order rate constants for the first two enzymes differ markedly.

The effects of added sucrose on the hydrolysis of benzylpenicillin catalysed by the RTEM β-lactamase were unexpectedly small (Fig. 3). This difference between RTEM β-lactamase on the one hand and β-lactamase I and PC1 β-lactamase on the other prompted us to test the effect of added sucrose on the hydrolysis of a relatively poor substrate by the RTEM enzyme. The hydrolysis of cephalosporin C was accelerated by added sucrose, as shown by the decrease in $K_m/k_{cat.}$. Thus the lack of effect of sucrose on the hydrolysis of benzylpenicillin could probably be ascribed to a fortuitous cancellation of

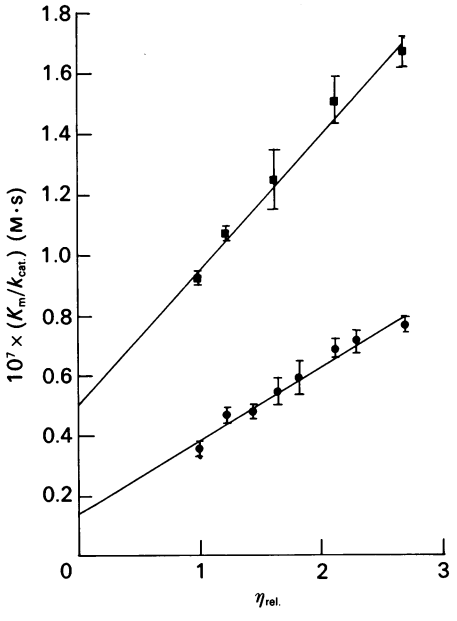

Fig. 2. Dependence of $K_m/k_{cat.}$ on relative viscosity for the hydrolysis of benzylpenicillin

Progress curves at pH 7 at 20 °C for the reaction in solutions containing added sucrose were measured spectrophotometrically and analysed by the half-time method. ●, β-Lactamase I; ■, PC1 β-lactamase. The error bars represent S.E.M. from at least three experiments.

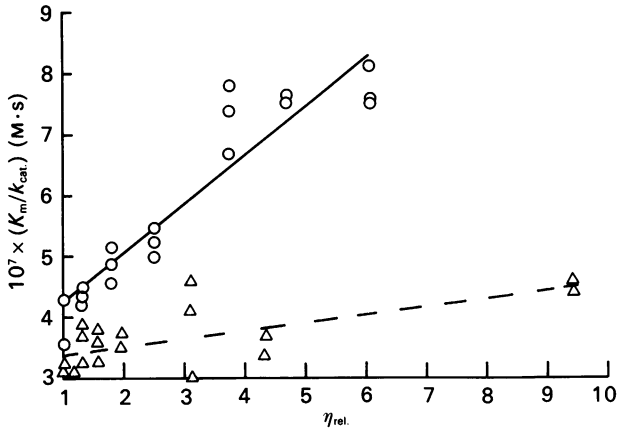

Fig. 3. Effect of sucrose or glycerol on the hydrolysis of benzylpenicillin by RTEM β-lactamase

Progress curves at pH 7 at 20 °C for the reaction in solutions containing added glycerol (○) or sucrose (△) were measured spectrophotometrically and analysed by the half-time method.

'accelerating' and 'retarding' effects on the RTEM β-lactamase. Moreover, when the viscosogen was changed to glycerol the expected retardation of the hydrolysis of the good substrate benzylpenicillin was observed (Fig. 3), whereas there was no effect when the poor substrate cephalosporin C was used. The results showed that, as with other β-lactamases, k_{-1} and k_{+2} were comparable.

The rate of hydrolysis of the cephalosporin nitrocefin by β-lactamase I was less affected by added viscosogen, but values of k_{+1} and k_{-1} of $7.9 \times 10^6 \pm 2 \times 10^6$ $M^{-1} \cdot s^{-1}$ and 240 ± 70 s^{-1} were obtained with the help of the value of k_{+2} from the single-turnover experiments described below.

Deuterium solvent isotope effects on the hydrolysis of benzylpenicillin

The effects on the acylation and deacylation rate constants of replacing ^1H_2O by ^2H_2O were investigated. It proved convenient to compare solvent ^1H_2O with 90% (v/v) ^2H_2O rather than with 100% ^2H_2O. The values of $^Dk_{+2}$ and $^Dk_{+3}$ in Table 2 were obtained by a short (linear) extrapolation to 100% ^2H_2O. The values obtained for β-lactamase I for $^Dk_{+2}$ and $^Dk_{+3}$ were 1.64 ± 0.2 and 1.88 ± 0.2 respectively, where $^Dk_{+2}$ is the ratio (k_{+2} in solvent ^1H_2O)/(k_{+2} in solvent ^2H_2O) and $^Dk_{+3}$ is defined similarly.

The $k_{cat.}$ for the hydrolysis of benzylpenicillin by β-lactamase I was measured in a series of isotopically mixed waters (Fig. 4). Since $k_{cat.}$ depends on both k_{+2} and k_{+3} (to roughly the same extent), the equation to describe these results must include solvent kinetic isotope effects on both acylation and deacylation. Hence the results, on the assumption of a single proton 'in flight', were fitted to eqn. (1), by the use of the simplex (polytope) method and non-linear regression (Press *et al.*, 1986). The values found for both $^Dk_{+2}$ and $^Dk_{+3}$ were 1.59 ± 0.1 (the value for k_{+2}/k_{+3} was 0.82). Thus fitting the $k_{cat.}$ data points to the rate constants for both acylation and deacylation having approximately the same isotope effect, and indeed the data in Fig. 4 are adequately fitted by a straight line, although a curve is not excluded.

Table 2. Solvent deuterium isotope effects on β-lactamase rate constants

The hydrolysis of benzylpenicillin was carried out in mixtures of ^2H_2O and ^1H_2O at 20 °C as described in the text. $^Dk_{+2}$ is the ratio (k_{+2} in solvent ^1H_2O)/(k_{+2} in solvent ^2H_2O), and $^Dk_{+3}$ is defined similarly.

β-Lactamase	$^Dk_{cat.}$	$^Dk_{cat.}/K_m$	$^Dk_{+2}$	$^Dk_{+3}$
β-Lactamase I	1.60±0.1	1.08±0.05	1.64±0.2	1.88±0.2
PC1 β-lactamase	2.16±0.2		2.10±0.3	1.60±0.2
RTEM β-lactamase	1.39±0.4	1.08±0.1	2.1±0.6	1.30±0.4

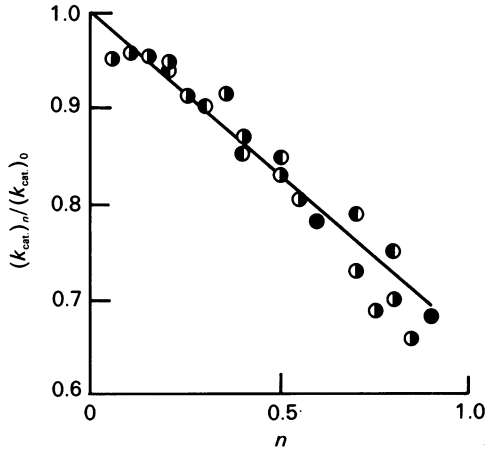

Fig. 4. Hydrolysis of benzylpenicillin in mixtures of ^1H_2O and ^2H_2O

The hydrolysis of 200 μM-benzylpenicillin by 2 nM-β-lactamase I was carried out at 20 °C; $k_{cat.}$ was determined from progress curves. Two series of experiments are shown, measurements in each mixture of waters being done in triplicate. The ordinate is $k_{cat.}$ in solvent relative to $k_{cat.}$ in ^1H_2O, and the abscissa is mole fraction of ^2H_2O.

Similar experiments carried out with PC1 β-lactamase showed that the fit of the data to eqn. (1) by non-linear regression gave a common value of 2.25±0.4 for $^Dk_{+2}$ and $^Dk_{+3}$.

β-Lactamase I and nitrocefin

The rate constants k_{+2} and k_{+3} for this chromophoric substrate were determined by comparison of single-turnover and steady-state experiments. The steady-state parameters (20 °C, pH 7, 50 mM-phosphate containing 0.5 M-NaCl and 0.1 mM-EDTA) were $k_{cat.}$ 20.8 s^{-1} and $k_{cat.}/K_m$ 3.48×10^5 $M^{-1} \cdot s^{-1}$ [the value of 62.3 $\mu M^{-1} \cdot s^{-1}$ in Martin & Waley (1988) was miscalculated]. The single-turnover experiments gave the results shown in Fig. 5, giving $k_{+2} = 29.5$ s^{-1} and K_s 81 μM, whence $k_{+3} = 1/(1/k_{cat.} - 1/k_{+2}) = 71$ s^{-1}. Thus here $k_{+2}/k_{+3} = 0.42$.

Rate constants for acylation and deacylation for β-lactamase I and penicillins

The quenched-flow method was applied to several penicillins (Table 3). In all cases the rate constant for acylation, k_{+2}, was greater than that for deacylation, k_{+3},

Table 3. Rate constants for acylation and deacylation for penicillins

The hydrolysis was carried out in 10 mM-sodium phosphate buffer, pH 7, containing 0.1 mM-NaCl and 10 μM-EDTA at 20 °C, and the reaction was measured by the change in absorbance at 240 nm. The apparent dissociation constant K' is $(k_{-1}+k_{+2})/k_{+1}$. The values in parentheses are standard errors of the mean derived from at least three experiments for the values immediately above.

Substrate	$k_{cat.}$ (s^{-1})	K_m (μM)	k_{+3} (s^{-1})	k_{+2} (s^{-1})	K' (μM)	$k_{cat.}/K_m$ ($\mu M^{-1} \cdot s^{-1}$)
β-Lactamase I						
Phenoxymethylpenicillin	2400	63	4510	5130	135	38.4
	(50)	(2)	(260)	(260)	(10)	(1.0)
Phenethicillin	1910	103	2700	6510	351	18.7
	(100)	(8)	(170)	(200)	(36)	(1.5)
L-Propicillin	631	48	708	5790	440	13.2
	(22)	(1)	(50)	(50)	(30)	(0.7)
D-Propicillin	1630	106	2100	7270	473	15.4
	(30)	(5)	(80)	(90)	(29)	(0.4)
Carbenicillin*	436	154	734	1070	378	2.84
	(25)	(4)	(55)	(60)	(34)	(0.2)
PC1 β-lactamase						
Phenoxymethylpenicillin	56.2	3.4	99	127	7.8	16.8
	(1.7)	(0.3)	(4)	(5)	(0.0)	(1.5)
Phenethicillin	34.7	5.7	52	107	17.4	6.06
	(0.3)	(0.2)	(3)	(6)	(1.6)	(0.15)

* Reaction at pH 7.7

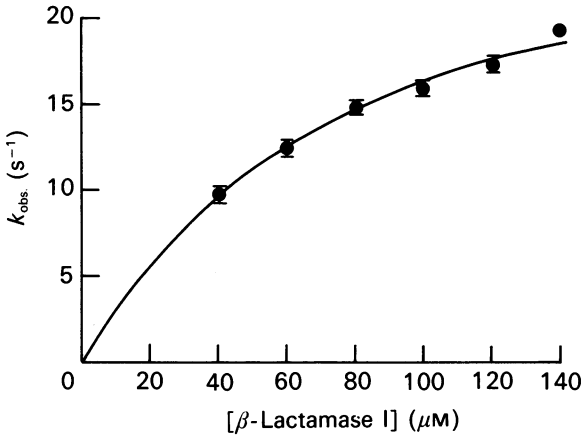

Fig. 5. Single-turnover hydrolysis of nitrocefin by β-lactamase I

The hydrolysis of nitrocefin (20 μM) at 20 °C in 50 mM-sodium phosphate buffer, pH 7, containing 0.5 M-NaCl and 0.1 mM-EDTA was measured at 500 nm in the stopped-flow instrument; the β-lactamase concentration was varied. The first-order rate constant for three to five experiments (except for the single experiment at 140 μM-β-lactamase I) is plotted against the concentration of enzyme. The error bars represent S.E.M. from at least three experiments.

but in no case was the difference large. For β-lactams with non-polar side chains k_{+2} varied less with structure than k_{+3} did; k_{+3} decreased as the bulk of the substituent in the branched side chain increased, as if the approach of the water molecule in the deacylation step were being hindered. The effect of varying the alkyl side chain on $k_{cat.}/K_m$ for penicillins has led to the conclusion that the interaction between the side chain and the enzyme is relatively weak (Buckwell *et al.*, 1988*a*). The K_m did not vary greatly, and is not, in general, a simple function of rate (or equilibrium) constants (see Appendix eqn. 2), but K', defined as $(k_{-1}+k_{+2})/k_{+1}$, did increase with the bulk of the substituent (Table 3). Both k_{+2} and k_{+3} were decreased in carbenicillin, which obeys Michaelis–Menten kinetics with β-lactamase I, in contradistinction to the behaviour with the PC1 β-lactamase (H. Christensen, M. T. Martin & G. S. Waley, unpublished work). Similarly, the presence of a carboxylate in the side chain of a cephalosporin hinders reaction (Buckwell *et al.*, 1988*b*).

Reaction of 6β-iodopenicillanate with β-lactamase I

The reaction of 3 μM-β-lactamase I with 30 μM-6β-iodopenicillanic acid at 20 °C was measured at 326 nm in the stopped-flow instrument; there was no evidence for more than one kinetically significant step, and the first-order rate constant was 0.18 s^{-1}, and hence the second-order rate constant was 6000 $M^{-1}\cdot s^{-1}$, which is in the usual range for class A β-lactamases (De Meester *et al.*, 1986). Measurements at three concentrations of ^2H_2O showed that the solvent kinetic isotope effect on this rate constant was 1.27±0.2. Opening the β-lactam ring, i.e. acylation, is (apart from re-arrangement) the only step here, and although the isotope effect was somewhat lower than the value for $^Dk_{+2}$ given in Table 2 it is not clear whether this is significant.

DISCUSSION

Relative magnitudes of rate constants for acylation and deacylation

The determination of the rate constants for acylation and deacylation for good substrates for β-lactamase I was reported previously (Martin & Waley, 1988). The present paper extends this to two related enzymes, namely the PC1 and RTEM β-lactamases. In all three instances the rate constants for acylation are approximately the same as those for deacylation, differing by no more than 2-fold. Pratt *et al.* (1988) reported that $k_{+2}/k_{+3} = (175\pm75)/(8.5\pm2.4) = 21\pm15$ for the PC1 β-lactamase and dansylpenicillin at pH 9, and concluded that the rate-determining step in hydrolyses of good substrates by PC1 β-lactamase was deacylation, at least at pH 9. Our conclusion differs: we believe that neither deacylation nor acylation is rate-determining for the many examples described above, at least at pH 7. Experiments carried out by R. Virden and co-workers also imply less than stoicheiometric accumulation of an acyl-enzyme (Virden *et al.*, 1978; R. Virden, personal communication). The difference between $k_{cat.}/K_m$ and k_{+2}/K_s that Pratt *et al.* (1988) found for dansylpenicillin may be accounted for as shown in the Appendix. It should be stressed that the comparability of k_{+2} and k_{+3} has only been established for penicillins as substrates; for some cephalosporins $k_{+2} < k_{+3}$ for β-lactamase I (Bicknell & Waley, 1985); for nitrocefin, a cephalosporin with an unusually reactive β-lactam ring, $k_{+2}/k_{+3} = 0.4$, as shown above.

Solvent kinetic isotope effects

The hydrolysis of benzylpenicillin by β-lactamase I was investigated in ^2H_2O in two ways. The first, more direct, way was to carry out the quenched-flow procedure in 90% ^2H_2O (at a suitable apparent pH) and to measure the observed F, and hence k_{+2} and k_{+3}. The results (Table 2) show normal solvent kinetic isotope effects (1.6 for k_{+2}, 1.9 for k_{+3}). Two other β-lactamases were studied similarly (Table 2), and the values for PC1 and RTEM β-lactamases extend the range downwards to 1.3 and upwards to 2.1. The mechanism of these values is now considered. In the first place, the mechanism of β-lactamase action clearly involves proton transfer. Since both acylation and deacylation have, within experimental error, the same solvent kinetic isotope effects, the values for $^Dk_{cat.}$ may be taken. The value for β-lactamase I (1.60±0.1) agrees with that reported (1.67) by Hardy & Kirsch (1984*b*). By way of comparison, 18 values for $k_{cat.}$ for several serine proteinases range from 1.4 to 4 (mean 2.7, standard deviation 0.6) (Venkatasubban & Schowen, 1984). This, and indeed the values for the other β-lactamases (Table 2), are towards the lower end of the range of values found for serine proteinases. It should be stressed that serine proteinases and serine β-lactamases are completely unrelated enzymes: the key histidine residue in the former is absent from the latter. Nevertheless, both reactions are hydrolyses of CO–N bonds, and so at some fundamental level similarities are expected. Indeed, both reactions are regarded as proceeding by way of tetrahedral intermediates, and stabilization of the anionic intermediate is probably crucial (see, e.g., Warshel *et al.*, 1989). Although there is little conformational change on acyl-enzyme formation in a related enzyme (Kelly *et al.*, 1989), β-lactamases are

often held to be 'floppy' enzymes, and if appreciable conformational change accompanies formation, or decomposition, of tetrahedral intermediates the magnitude of the solvent kinetic isotope would be decreased. The main conclusion from the results given in Table 2 is that the magnitudes of the solvent kinetic isotope effect were comparable for acylation and deacylation, for all three β-lactamases. This suggests that the mechanisms for proton transfer in acylation and deacylation may not differ much; the similar pH-dependences of k_{+2} and k_{+3} (Martin & Waley, 1988) are consistent with this view.

Further information can be obtained from proton inventories, reactions carried out in mixtures of ^1H_2O and ^2H_2O (Schowen & Schowen, 1982; Venkatasubban & Schowen, 1984). The background was outlined above in the Materials and methods section. A particularly illuminating example of the method applied to a hydrolytic enzyme has been provided by Szawelski & Wharton (1981). In favourable cases the number of protons 'in flight' in a transition state can be determined. If two protons were 'in flight' (in both transition states) then the terms linear in n would be replaced by quadratic terms. Unfortunately, a weakness of the method of proton inventories is that discrimination between these rival possibilities is not practicable when the isotope effects are 2 or less unless the data are unusually precise; thus the precision required for a solvent isotope effect of 2 with one or two protons 'in flight' has been given as 2.8% and 1.1% respectively (Schowen, 1978). The results reported here do not permit discrimination between mechanisms with one or two protons 'in flight'.

Hydrolysis of a good substrate is partly diffusion-controlled

The high values for $k_{cat.}/K_m$ found when β-lactamases hydrolyse good substrates had suggested that the rate might be partially diffusion-controlled (Abraham & Waley, 1979), and that this was clearly demonstrated by Hardy & Kirsch (1984*a*). The method that they used was to increase the viscosity of the medium by adding glycerol, sucrose or polymers such as Ficoll. There is, of course, the possibility that the viscosogenic agent might affect the rate of the reaction other than by increasing the viscosity. This was excluded for β-lactamase I by comparison of the effects on several substrates: the viscosogenic agents had little or no effect on $k_{cat.}/K_m$ for poor substrates (Hardy & Kirsch, 1984*a*). For good substrates, however, the addition of glycerol or sucrose did bring about a decrease in $k_{cat.}/K_m$. Ficoll had relatively little effect on the values of the kinetic parameters found for hydrolyses catalysed by β-lactamase I (Hardy & Kirsch, 1984*a*), but this is not unexpected as polymers do not increase the 'microviscosity' of the solution, the extent to which the medium impedes the motion of a (small) molecule (Blacklow *et al.*, 1988).

Our values of k_{-1}/k_{+2} for benzylpenicillin were of the order of 1 for all three enzymes (0.57 for β-lactamase I, 1.1 for PC1 β-lactamase and 4.2 for RTEM β-lactamase). Hardy & Kirsch (1984*a*) found values of 1.1 to 2.3 for β-lactamase I (depending on the conditions); the significant point was that their values of the order of 1 for the good substrate benzylpenicillin contrasted with values of about 30 for a poor substrate, cephaloridine. A value of k_{-1}/k_{+2} of the order of 1 means that the 'chemical' step of acylation has been so accelerated by the enzyme that its rate is comparable with that of the 'physical' step of dissociation of the non-covalent enzyme–substrate complex. This was also shown by plots (not shown) of the relative $k_{cat.}/K_m$ against the relative viscosity for the data shown in Fig. 2; the slopes of these linear plots indicated that reactions catalysed by β-lactamase I, PC1 β-lactamase and RTEM β-lactamase were about 80%, 50% and 10% diffusion-controlled respectively.

There used to be debate about whether K_m could be taken as equal to the dissociation constant of (k_{-1}/k_{+1}) of the enzyme–substrate complex. We see here that K_m does turn out to have approximately this value for benzylpenicillin (Table 1), as it must when k_{-1}, k_{+2} and k_{+3} are approximately equal (see the Appendix). All four rate constants appear in the expression for K_m, which suggests that correlations between K_m and structure may be misleading unless the relative magnitudes of the first-order rate constants have been determined.

β-Lactamases are fully efficient enzymes

The conditions for maximum efficiency of an enzyme have been much discussed (Albery & Knowles, 1976*b*,

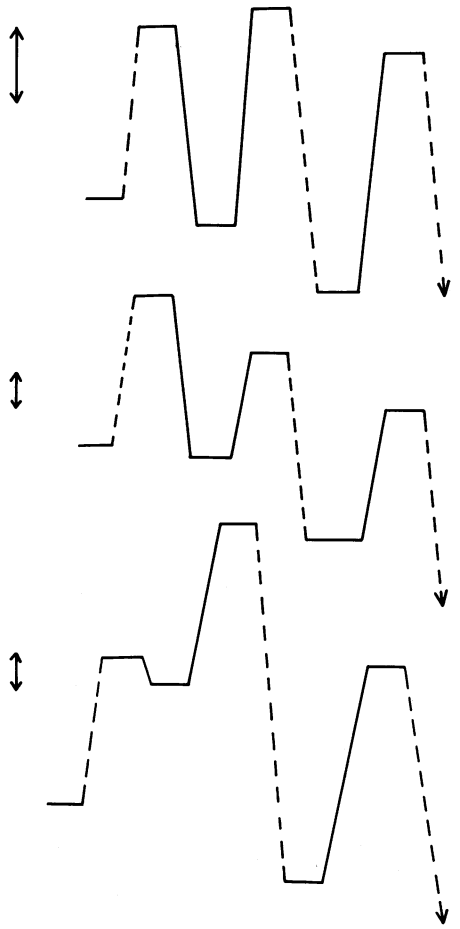

Fig. 6. Free-energy profile for the hydrolysis of benzylpenicillin

Broken lines indicate uncertainty about the level of the acyl-enzyme intermediate (the second minimum) and the arbitrary position of E+S, which depends on the concentration of substrate. The lowest profile is that of RTEM β-lactamase, the middle one that of β-lactamase I and the uppermost one that of PC1 β-lactamase. The arrow signifies 1 kJ/mol.

1977; Chin, 1983; Ellington & Benner, 1987) and two main cases distinguished. In the first ('reversible') case, there are comparable concentrations of product and substrate present in the reaction in the cell; in the second case, and β-lactamases belong here, the back reaction may be neglected, either because the reaction is effectively irreversible or because the concentration of product is negligible. β-Lactamase I and the PC1 enzyme are extracellular (RTEM β-lactamase is periplasmic) and the concentration of substrate *in vivo* is not well-defined. Moreover, the conditions under which they function naturally differ widely; *B. cereus* is a soil organism, and *S. aureus* and *E. coli* are parasites. It is remarkable that, for each enzyme, k_{-1}, k_{+2} and k_{+3} are comparable but the absolute values for the enzymes differ appreciably. Thus the free-energy profiles are similar, and show 'matched peaks' (Fig. 6). For a reaction that operates far from equilibrium, it may be shown that maximum catalytic efficiency demands that, in the mechanism of Scheme 1, $k_{+2} = k_{+3}$ (J. J. Burbaum, R. T. Raines, W. J. Albery & J. R. Knowles, unpublished work). Moreover, the fact that good substrates react at near the diffusion-controlled limit means that the transition state for enzyme–substrate combination is matched with that for acylation, as is shown by k_{-1} being comparable with k_{+2} and k_{+3}. Thus, by these criteria, these β-lactamases are nearly fully efficient.

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APPENDIX

Significance of $k_{cat.}/K_m$ and K_m in the acyl-enzyme mechanism

It may readily be shown that, in the widely applicable acyl-enzyme mechanism of Scheme 1 of the main paper, the reciprocal of the specificity constant is given by:

$$\frac{K_m}{k_{cat.}} = \frac{1}{k_{+1}}\left(1+\frac{k_{-1}}{k_{+2}}\right) = \frac{K_s}{k_{+2}} + \frac{1}{k_{+1}} \qquad (1)$$

where K_s is the dissociation constant of the non-covalent enzyme–substrate complex ES (i.e. k_{-1}/k_{+1}). Thus comparison of $K_m/k_{cat.}$ obtained from steady-state experiments and K_s/k_{+2} obtained from single-turnover experiments can give a value for k_{+1}, the rate constant for enzyme–substrate combination. From eqn. (1) it is clear that the equality of $k_{cat.}/K_m$ and k_{+2}/K_s does not hold unless k_{+1} is much larger than k_{+2}/K_s; this is more often assumed than tested, and we show that it does not hold for the reactions studied in the main paper.

The significance of K_m has so often been discussed that it is surprising that one condition under which K_m equals K_s does not appear to have attracted comment. In the acyl-enzyme mechanism of Scheme 1 of the main paper it may readily be shown that K_m is given by:

$$K_m = \left(\frac{k_{-1}+k_{+2}}{k_{+1}}\right)\left(\frac{k_{+3}}{k_{+2}+k_{+3}}\right) \qquad (2)$$

The familiar condition for K_m equalling K_s is $k_{-1} \gg k_{+2}$ together with $k_{+3} \gg k_{+2}$, but an alternative condition is seen as follows. Were k_{-1} to equal k_{+3} then the numerator in the first term in eqn. (2) would cancel the denominator in the second (irrespective of the relative magnitudes of k_{+2} and k_{+3}) and then $K_m = k_{+3}/k_{+1}$, i.e. $k_{-1}/k_{+1}(= K_s)$ here. Although this alternative condition may seem unduly restrictive, the theme of the main paper is that for several β-lactamases (and, by extension, for all highly efficient enzymes obeying the acyl-enzyme mechanism) all the first-order rate constants have approximately the same value (and hence k_{-1} is approximately equal to k_{+3}). Hence this second condition may in fact be quite often fulfilled, at least approximately.

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